Tumor Suppressor Menin Represses Paired Box Gene 2 Expression via Wilms Tumor Suppressor Protein-Polycomb Group Complex*[S]

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Tumor suppressor menin, the product of the *MEN1* gene, plays a key role in controlling histone 3 lysine 4 trimethylation (H3K4me3) and gene transcription, which can regulate proliferation, apoptosis, and differentiation. However, little is known as to whether menin controls gene expression and cell proliferation and survival via regulating Polycomb group (PcG) protein complex/H3K27me3. Here we show that menin specifically represses transcription factor Paired box gene 2 (*Pax2*) through PcG-mediated H3K27me3 and Wilms tumor suppressor protein (WT1), a zinc finger domain-containing DNA-binding protein. Menin does not directly bind to the *Pax2* locus, instead, it up-regulates WT1 expression. WT1 recruits PcG complex to the *Pax2* promoter and represses expression of *Pax2* through PcG-dependent H3K27me3. Moreover, WT1 also interacts with DNA methyltransferase 1 (DNMT1), and recruits DNMT1 to the *Pax2* promoter, resulting in hypermethylation of CpG in the *Pax2* promoter. Together, these studies have uncovered a novel epigenetic mechanism whereby menin regulates H3K27me3 and promoter DNA methylation via WT1 and suggest that WT1 protein plays an important, yet previously unappreciated role in regulating the function of the menin/PcG axis, H3K27 methylation, and DNA methylation, resulting in repression of gene transcription.

Epigenetic regulation of gene expression is necessary for the maintenance of cell fates and developmental programs (1, 2). Lysine methylation of histones is recognized as an important epigenetic indexing system (1, 2). Among them, the trithorax group (trxG)[3] and Polycomb group gene (PcG) are thought to play an essential role in these epigenetic programming events (2). TrxG and PcG proteins bind to specific regions of target gene promoters and direct the posttranslational modifications of histones, resulting in activating or silencing of gene expression, respectively (2, 3). For instance, mixed lineage leukemia (MLL), the human homolog of *Drosophila* trithorax, trimethylates histone 3 lysine 4 (H3K4me3) and maintains transcriptional activity (4, 5). MLL catalyzes H3K4 methylation and increases expression of multiple *Hox* genes, which are required for embryonic development, hematopoiesis, and leukemogenesis (6–10).

In contrast, PcG proteins are epigenetic gene silencers that play important roles in embryonic development, maintenance of embryonic stem cells, and tumorigenesis (1, 11). PcG proteins form various protein complexes including Polycomb repressive complex 2 (PRC2), which includes Enhancer of Zeste homolog 2 (EZH2) and its regulatory protein SUZ12 (11). EZH2 is also a chromatin associating protein with a conserved SET domain. However, unlike the SET domain in MLL that methylates H3K4, the SET domain of EZH2 specifically methylates H3K27, and the methylated H3K27 can be recognized by other specific binding proteins to compress chromatin structure, leading to repression of gene transcription, such as *Hox* family genes (1, 11, 12).

Menin is a nuclear protein encoded by the *MEN1* gene, whose loss of function results in human tumor syndrome known as multiple endocrine neoplasia type 1 syndrome (MEN1) (13). In a mouse model with *Men1* disruption, heterozygous mice develop features remarkably similar to those of the human disorder, demonstrating a critical role for menin in suppressing the development of MEN1 (14). Menin has been shown to associate with a multimember protein complex whose composition is highly similar to that of the SET1-HMTase complex of yeast and humans (7–9, 15–17). Menin interacts with trxG family proteins in an HMTase complex including trxG proteins MLL1/MLL2, Rbbp5 (retinoblastoma binding protein 5), WDR5 (WD repeat domain 5), and ASH2 (absent, small, or homeotic), and promotes methylation of H3K4 at the $p27^{Kip1}$ (*p27*) and $p18^{Ink4c}$ (*p18*) promoter loci, but the patient-derived menin point mutants failed to bind to the promoter in islet cells (16). These findings suggest that menin maintains transcription of cell cycle regulators partly by promoting H3K4me3 to suppress development of MEN1.

Interestingly, menin retains the ability to associate stably with oncogenic MLL fusion proteins through a high affinity N

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and a table.
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[3] The abbreviations used are: trxG, trithorax group; DNMT1, DNA methyltransferase 1; EZH, Enhancer of Zeste homolog; H3K27me3, histone 3 lysine 27 trimethylation; IHC, immunohistochemistry; MEF, mouse embryonic fibroblast; MEN1, multiple endocrine neoplasia type 1 syndrome; MLL, mixed lineage leukemia; *Pax2*, Paired box gene 2; PcG, Polycomb group; PP, primer pair; PRC, Polycomb repressive complex; qRT-PCR, quantitative RT-PCR; WT1, Wilms tumor suppressor protein; HMTase, histone methyltransferase; SET, Su(var)3-9, Enhancer of Zeste, and trithorax.

terminus, and this interaction is required for maintenance of *Hox* family gene expression and initiation of MLL fusion protein-mediated leukemogenesis and myeloid transformation (9). Recent studies show that menin is a molecular adaptor that links the MLL HMTase with lens epithelium-derived growth factor (18). In addition, c-Myb promotes MLL-associated leukemogenesis by binding MLL through menin (7). We have found that menin recruits both wild-type MLL and oncogenic MLL-AF9 fusion protein to loci of Hox genes to promote their expression through trimethylation of H3K4 and dimethylation of H3K79 in MLL-AF9 induced leukemogenesis (8). Together, these findings strongly suggest an epigenetic function of menin.

Currently, it is poorly understood whether menin function is associated with repressive epigenetic markers such as H3K27 methylation. In our previous report, we found that menin represses transcription of growth factor pleiotrophin at least in part through PcG-mediated H3K27me3 (19). However, the precise mechanisms underlying the functional interaction between menin and PcG in H3K27 methylation-mediated repression of gene transcription remain unclear.

In the present study, we investigated the molecular and biochemical mechanisms whereby menin regulates H3K27 methylation. Our results show that menin represses gene transcription of Paired box gene 2 (*Pax2*), which belongs to a family of genes that play a critical role in the formation of tissues and organs during embryonic development (20). However, no evidence suggests that menin directly bound to the *Pax2* locus and directly interacted with PcG proteins. Further studies indicate that menin up-regulates expression of Wilms tumor suppressor protein (WT1), and WT1 recruits PcG proteins and DNMT1 to the *Pax2* promoter locus, represses *Pax2* expression through trimethyl-H3K27 modification and CpG hypermethylation at this region. These findings suggest a novel means whereby menin epigenetically represses its target genes by recruiting PcG complex and increasing transcriptionally repressive H3K27me3.

EXPERIMENTAL PROCEDURES

Cell Culture and Gene Transfection—Generation and culture of $Men1^{-/-}$ mouse embryonic fibroblast (MEF) cell lines were described previously (21). In the present study, $Men1^{-/-}$ MEFs were transduced with either pMX-puro vector or menin-expressing retroviruses and selected with puromycin. 293-FT, GP2–293, wild type MEF cells, and β-TC-6 insulinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) and supplemented with 10% (v/v) or 15% (v/v) (β-TC-6) fetal bovine serum (FBS) (Hyclone), $1\times$penicillin-streptomycin (100 units/ml–100 μg/ml) (Invitrogen) and 4 mM L-glutamine (Invitrogen). Plasmids were introduced into cells by polyethylenimine (PEI)-mediated transfection as described previously (19).

Construction of WT1-expressing Plasmid, EZH2, SUZ12, and WT1 shRNA Plasmids and Production of Recombinant Retroviruses—The WT1 cDNA was inserted into the HindIII/NotI site of the retroviral vector pLNCX2 (631508; Clontech) from a pcDNA3.1-WT1 (gift from Dr. Mark D. Minden). The sequences of EZH2, SUZ12, and WT1 shRNA were designed by Clontech RNAi designer and were constructed into the retro-

viral vector RNAi-Ready pSIREN-RetroQ (632456; Clontech). Recombinant retroviruses were packaged using GP2-293 cells according to the BD Retro-X™ Universal Packaging System protocol (BD Biosciences Clontech).

Western Blotting—The Western blotting was performed as described previously (19). Antibodies are listed in the supplemental table.

Immunoprecipitation (IP) Assays—IP assays were performed as described previously (22). Briefly, the harvested cells were lysed with 1% Nonidet P-40 and incubated with a primary antibody (supplemental table) or normal rabbit IgG. Protein A (DAM1407372A; Millipore) or protein G-agarose beads (sc-2002; Santa Cruz Biotechnology) were added. Bound proteins were collected by centrifugation, washed, and analyzed by Western blotting. IgG served as negative control.

Real-time Quantitative RT-PCR—Quantitative reverse transcription-PCR (qRT-PCR) was performed as described previously (19), using an ABI PRISM 7300 detection system with primers listed in the supplemental table. RT-PCRs were repeated at least for three times.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed as described previously with certain modifications (19). Briefly, 1×10^6 MEFs were treated with 1% formaldehyde for 10 min at 37 °C, followed by pulsed ultrasonication to shear cellular DNA. ChIP assays were then carried out with the indicated antibodies according to the protocol of the ChIP Assay kit (DAM1442485; Millipore). After overnight incubation with the antibodies, protein A- or protein G-agarose beads were added. The cross-links between nuclear proteins and genomic DNA were reversed, and DNA pulled down by the antibody was purified by phenol/chloroform extraction. The antibodies and primer pairs (PP) sequences for ChIP assays are shown in the supplemental table.

Immunofluorescent Staining and Immunohistochemistry (IHC)—Immunofluorescent staining and IHC were performed using an affinity-purified anti-menin (1:200 dilution) antibody (19), anti-WT1 (1:50 dilution) or anti-Pax2 (1:80 dilution) antibodies for MEFs. $Men1^{\Delta N3}$-*MACROS BELOW ARE FOR THE VISUAL*$^{-8/+}$ mice heterozygous for the $Men1$ locus ($Men1^{+/-}$) were a gift from Dr. Francis Collins at NHGRI and were maintained on a 129S6/SvEvTac background (14). The 6-month mice were killed, and expression of WT1 in kidney was determined using IHC. For immunofluorescent staining, the sections were incubated with FITC-conjugated swine anti-rabbit antibody (1:80 dilution). Nuclei were counterstained with DAPI, and the stained cells were analyzed and photographed under a confocal microscope (Olympus FV1000). IgG was used as negative control. The antibodies are listed in the supplemental table.

DNA Methylation Assay—Primers for detecting DNA methylation were designed by a Web-based CpG island design program (Urogene) and are listed in the supplemental table. Genome DNA was extracted by using the CpGenome DNA Modification kit according to the protocol (PSO1443610; Millipore). The primers were used for PCR amplification, and the outputs were inserted into T carrier and transfected them into *Escherichia coli*. Selected colonies were sequenced and examined, and the methylation state of each CpG was identified.

RESULTS

Menin Specifically Suppresses Pax2 Expression—In our previous studies, we found that menin suppressed transcription of growth factor pleiotrophin partly through trimethylation of H3K27 (19), but the precise biochemical mechanism remained poorly understood. To elucidate the role of menin and PcG-mediated H3K27me3 modification in regulating expression of target genes, we further investigated the role of menin in regulating *Pax2* gene transcription, which was identified as a menin-repressed gene in our previous microarray analysis (23). *Pax* genes consist of tissue-specific transcription factors that contain the paired domain and homeodomain and are important for the specification of distinct tissues and epimorphic limb regeneration in mammals (24). Among them, Pax2 expresses in the developing pancreas, central nervous system, and spinal cord. Recently, results from a ChIP-sequencing assay show that SUZ12, a member of the PcG, binds to the promoter of the *Pax* family genes including *Pax2* in embryonic stem (ES) cells (25).

These results suggest a possibility that menin and PcG act together in regulating transcription of certain target genes. To test this possibility, the *Men1*-null MEFs were transduced with either pMX-puro vector or menin-expressing retroviruses and selected with puromycin, designated as *Men1*-knock-out (KO) and *Men1*-reexpressing (*Men1*) MEFs. Real-time qRT-PCR was used to determine mRNA levels of *Pax2*, *Pax5*, and *Pax8*, which

belong to the subgroup II of the Pax family (26). Interestingly, ectopic menin expression reduced the mRNA levels of *Pax2* and *Pax5*, but not *Pax8* in MEFs (Fig. 1*A*). Next, Western blotting results also indicate that menin expression substantially reduced the Pax2 protein level, but did not affect the expression of Pax6 (Fig. 1*B*), a member of Pax4/6 subgroup that is required for the development of all pancreatic endocrine cells and transactivates the glucagon, insulin, and somatostatin genes (27–29). We further determined Pax2 expression in the *Men1*-null and *Men1*-reexpressing MEFs in an exponential growth phase, using IHC and immunofluorescent staining. As illustrated in Fig. 1*C* and supplemental Fig. S1, the *Men1*-null MEFs failed to show menin staining, but displayed an obvious nucleus staining for Pax2. Conversely, *Men1*-reexpressing MEFs showed a clear staining for nuclear menin, but a reduction of nuclear Pax2 staining. Similar results were also found in another pair of menin-null and menin-expressing MEFs (data not shown). Moreover, consistent with this observation, Pax2 expression also up-regulated in *Men1*-null MEF cells compared with wild type MEF (data not shown). Collectively, these results indicate a pivotal role for menin in down-regulating *Pax2* mRNA and protein expression.

Menin Increases the Trimethyl-H3K27 Modification on the Pax2 Promoter Locus—Currently, little is known as to whether menin is involved in regulating repressive histone modifications to suppress transcription of *Pax2*. To determine whether menin has a role in regulating target genes in concert with PcG, we first performed ChIP assays with *Men1*-null and *Men1*-reexpressing MEFs to detect whether menin affects histone modifications at the *Pax2* locus. We designed three distinct pairs of primers for ChIP analysis at the *Pax2* promoter (Fig. 2*A*). ChIP assays showed that *Men1* excision reduced H3K27me3 at the *Pax2* locus (Fig. 2*B*), but failed to affect the level of H3K4me3 or acetyl-H3 (supplemental Fig. S2, *A and B*). It raises an interesting possibility that PcG-mediated H3K27 methylation possibly participates in menin-regulated suppression of *Pax2* gene transcription. To explore this possibility further, we examined the
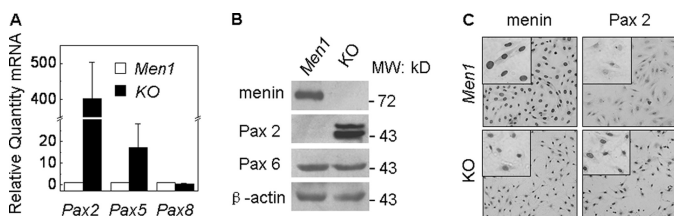

FIGURE 1. **Menin specifically represses Pax2 expression.** *A*, increased *Pax2* and *Pax5*, but not *Pax8* mRNA levels in *Men1*-null (*KO*) MEFs compared with *Men1*-reexpressing (*Men1*) MEFs detected by qRT-PCR. *B*, efficiency of menin silencing and effect on up-regulation of Pax2 protein determined by Western blotting. *C*, IHC detection of menin and Pax2 expression and localization in MEFs. Original magnification, ×100 and ×400.
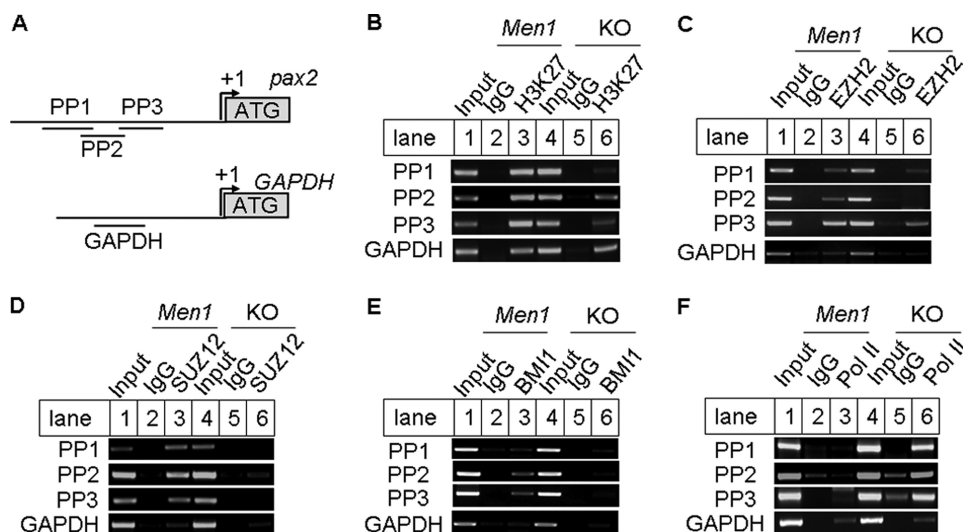

FIGURE 2. **Menin increases H3K27me3 modification at the *Pax2* promoter.** *A*, schematic representation of the mouse *Pax2* and GADPH gene loci and primer pairs (*PP*) used for ChIP assays. *B–F*, ChIP assays using one of the antibodies against H3K27me3, EZH2, SUZ12, BMI1, RNA polymerase II, and control IgG in *Men1*-null and *Men1*-reexpressing MEFs. PCR was carried out using primers for each amplicon.
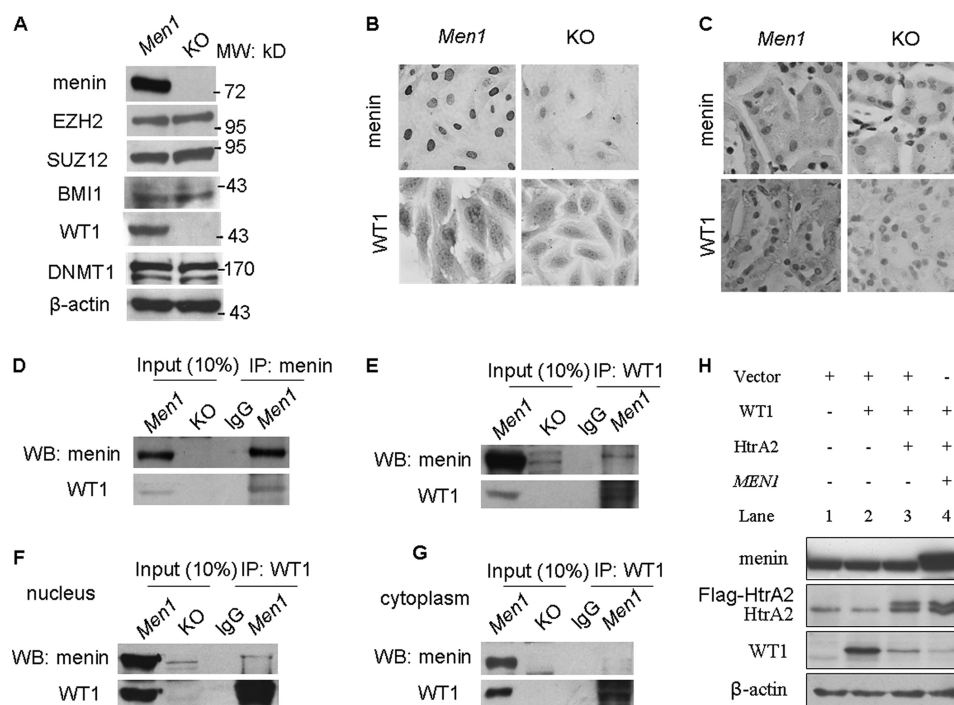
FIGURE 3. **Menin up-regulates WT1 expression and interacts with WT1.** *A*, expression of EZH2, SUZ12, Bmi1, DNMT1, and WT1 in MEFs determined by Western blotting. *B*, IHC detection of menin and WT1 expression and localization in MEFs. Original magnification, ×400. *C*, IHC detection of menin and WT1 expression in the kidney of wild-type and *Men1* heterozygous knock-out mice. Original magnification, ×400. *D* and *E*, total protein lysates of *Men1*-null and *Men1*-reexpressing MEFs used for endogenous co-IP with the indicated antibodies to menin, WT1, and IgG as a control, followed by Western blotting (*WB*) for WT1 and menin. *F* and *G*, nucleus and cytoplasm protein lysates of *Men1*-null and *Men1*-reexpressing MEFs used for endogenous co-IP with the antibody to WT1 and IgG as a control, followed by Western blotting of menin and WT1. *H*, ectopic expression of WT1 and/or HtrA2 without or with menin determining their effects on WT1 protein level in 293T cells.

impact of *Men1* excision on binding of other PcG proteins of the H3K27-methylating complex using ChIP assays. The results showed that EZH2, SUZ12, and BMI1 bound to the *Pax2* locus (Fig. 2, *C–E*, *lanes 3*). Notably, loss of menin abrogated EZH2, SUZ12, and BMI1 binding to the *Pax2* locus (Fig. 2, *C–E*, *lanes 6*). RNA polymerase II is associated with transcription initiation, elongation, and termination. The PRC complex has a direct role in restraining RNA polymerase II processivity at bivalent genes (30). As expected, the ChIP assay indicates that RNA polymerase II bound to the *Pax2* promoter (Fig. 2*F*, *lane 3*), and loss of menin enhanced detection of RNA polymerase II at the *Pax2* locus (Fig. 2*F*, *Lane 6*). Together, these experiments demonstrate a crucial role for menin in PcG protein recruitment and H3K27me3 at the *Pax2* locus for *Pax2* repression.

Menin Up-regulates WT1 Expression and Interacts with WT1— To explore further whether menin directly regulates *Pax2* transcription and histone tail modification, we performed a ChIP assay with an anti-menin antibody. However, we failed to show that menin binds directly to the *Pax2* locus (supplemental Fig. S2C). This result raises the possibility that menin represses transcription of *Pax2* via an indirect mechanism. Thus, we first examined whether menin regulates PcG protein expression. Western blotting showed that menin excision did not affect expression of EZH2, SUZ12, and BMI1 (Fig. 3*A*). WT1, the Wilms tumor suppressor gene, encodes multiple isoforms of DNA-binding protein with zinc finger domain and acts as a crucial transcriptional regulator for normal kidney development and renal tumorigenesis (20, 31, 32). Recently, decreased or lost expression of WT1 mRNA in primary pancreatic tumors

was also reported (33). Ryan *et al.* found that *Pax2* is likely a target gene of WT1 and that there is an inverse relationship between expression of WT1 and *Pax2*, suggesting transcriptional repression of *Pax2* by WT1 (32). Based on the aforementioned observations, we examined whether menin regulates Pax2 expression through WT1. Western blotting results clearly indicate that WT1 protein expression was markedly reduced in *Men1*-null MEFs (Fig. 3*A*). *Men1*-reexpressing MEFs showed a clear staining for nuclear menin and increased WT1 expression in nuclear and cytoplasm, as determined by IHC (Fig. 3*B*). Because WT1 is essential for renal development and tumorigenesis, we further investigated the effect of menin loss on WT1 expression in kidneys of $Men1^{+/-}$ mice. The IHC staining for WT1 indicated that WT1 expression was also reduced in the kidneys of $Men1^{+/-}$ mice (Fig. 3*C*). To elucidate how menin up-regulates WT1 expression, we turned our attention to the impact of menin on regulating WT1 transcription. Interestingly, *Men1* excision did not reduce WT1 mRNA expression, as determined by RT-PCR (data not shown). ChIP assays also showed that menin did not affect the levels of H3K4me3 and acetylated H3 at the *WT1* promoter (supplemental Fig. S3). These results suggest that menin up-regulated *WT1* expression is likely through posttranscriptional regulation. Next, we performed co-immunoprecipitation (co-IP) from extracts of MEFs and found that endogenous WT1 was co-immunoprecipitated with menin (Fig. 3*D*). The interaction between menin and WT1 was further confirmed by reverse co-IP of menin with an anti-WT1 antibody (Fig. 3*E*). Menin was also detected in WT1 immunoprecipitates with nuclear extracts (Fig. 3*F*), but not in
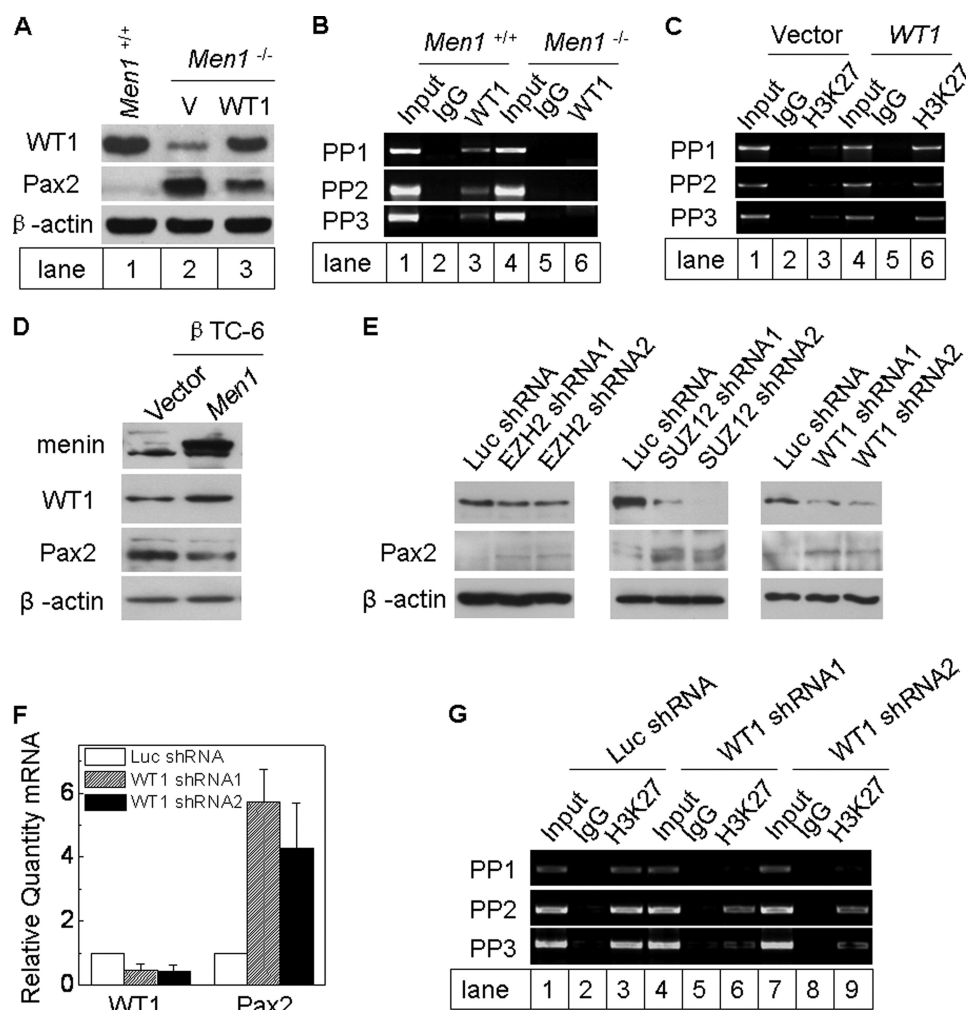
FIGURE 4. **WT1 and PcG proteins are essential for menin-mediated repression of Pax2 expression.** *A, Men1*-null MEFs were transduced with either pLNCX2 control vector or WT1-expressing retroviruses. The resulting cells and *Men1*-reexpressing MEFs were processed to determine Pax2 protein levels by Western blotting. *B* and *C*, ChIP assays, with three distinct amplicons, were used for detecting WT1 binding at the *Pax2* promoter (*B*) and H3K27me3 (*C*) in MEFs. *D*, expression of WT1 and Pax2 was determined by Western blotting in stably *Men1*-overexpressing β-TC-6 insulinoma cells. *E*, wild type MEF cells were tranduced with either RNAi-Ready pSIREN-RetroQ luciferase shRNA or EZH2, SUZ12, and WT1 shRNAs retroviruses, selected with puromycin (2 μg/ml). The stable cell lines were used for Western blotting with antibodies to β-actin, EZH2, SUZ12, WT1, and Pax2. *F*, efficiency of WT1 knockdown and increased *Pax2* mRNA expression was detected by real-time qRT-PCR in MEFs. *G*, decreased H3K27 trimethylation level at *Pax2* promoter loci was determined by ChIP assays.

cytoplasm (Fig. 3*G*). These results suggest that menin interacts with WT1 in the nucleus. Recently, Hartkamp *et al.* have reported that serine protease HtrA2 is a WT1-binding partner and cleaves WT1 at multiple sites (34). Our results, coupled with this recent report, raise a possibility that menin up-regulates WT1 expression through protecting WT1 from cleavage by proteolytic enzymes. To dissect the potential relationship between menin and WT1 further, we ectopically expressed WT1 and/or HtrA2 with or without menin to determine the impact of menin on the WT1 protein level in transfected 293T cells. Our result shows that ectopic expression of HtrA2 indeed reduced WT1 protein expression (Fig. 3*H*, *lane 3*), but the overexpressed menin failed to suppress HtrA2-mediated cleavage or reduction of full-length WT1 protein (Fig. 3*H*, *lane 4*). These results suggest that menin up-regulated WT1 expression is independent of proteolytic degradation mediated by HtrA2. The above experiments indicate that menin can specifically interact with WT1, but how menin regulates WT1 protein remains to be investigated further.

WT1 and PcG Proteins Are Essential for Menin-mediated Repression of Pax2 Expression—To examine the necessity of WT1 for Pax2 expression, the *Men1*-null MEFs were transduced with either pLNCX2 vector or WT1-expressing retroviruses, and the resulting cells were used for Western blotting. Down-regulated Pax2 expression following ectopic WT1 expression was observed in WT1-expresssing *Men1*-null MEFs (Fig. 4*A*, *lane 3*). WT1 is a zinc finger-containing transcription factor known to bind selectively to DNA or mRNA targets (20). Therefore, we examined whether WT1 bound to the *Pax2* promoter. ChIP assays clearly showed that WT1 bound to the *Pax2* promoter in *Men1*-reexpressing MEFs, but not in *Men1*-null MEFs (Fig. 4*B*, *lanes 3* and *6*). We further determined whether WT1 affects H3K27me3 on the *Pax2* locus. Notably, the H3K27me3 level was increased in WT1-overexpressing *Men1*-null MEFs at the *Pax2* promoter (Fig. 4*C*, *lanes 3* and *6*). Because menin plays an important role in suppressing β-cells, we also tested the role of menin in regulating β-cells. We found that menin up-regulated WT1 and down-regulated Pax2
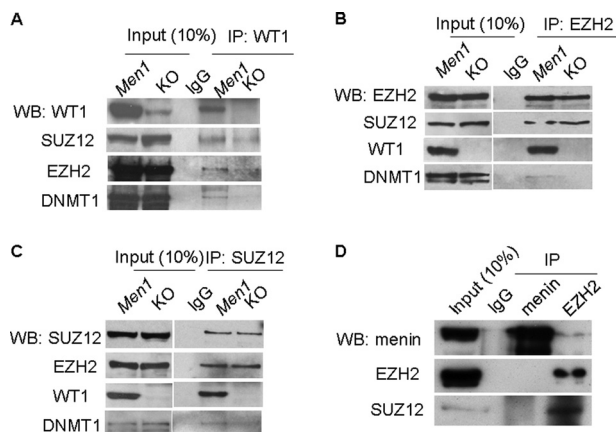
FIGURE 5. **WT1 interacts with PcG complex.** *A,* co-IP assays using MEF lysates show specific co-IP of WT1 with SUZ12, EZH2, and DNMT1. *B* and *C,* co-IP of endogenous proteins was performed with the indicated antibodies to EZH2 and SUZ12 followed by Western blotting (*WB*) for WT1, EZH2, SUZ12, and DNMT1. *D,* co-IP was performed with antibodies to menin and EZH2 followed by Western blotting for menin, EZH2, and SUZ12. Where indicated, 10% of input is presented as loading control.

FIGURE 6. **WT1 recruits DNMT1 to facilitate CpG methylation of the *Pax2* promoter locus.** *A, Men1*-null and *Men1*-reexpressing MEFs lysates used for co-IP with antibody to DNMT1 followed by Western blotting for WT1, EZH2, and Bmi1. *B,* ChIP assays using the antibody against DNMT1, and IgG as a control in *Men1*-null and *Men1*-re-expressing MEFs. PCR was carried out using primers for each amplicon. *C,* two pairs of methylation primers P1 and P2 in CpG islands from −1 to −2000 of the *Pax2* promoter. *D,* 10 selected colonies for sequencing and alignment. *White* and *black circles* represent unmethylated and methylated CpGs, respectively. 10% of input is presented as loading control.

expression in β-TC-6 insulinoma cells (Fig. 4*D*). Similar results were detected in another pair of vector- and menin-overexpressing-β-TC-6 insulinoma cells ([supplemental Fig. S4](#)). Next, we examined whether knockdown of PcG proteins and WT1 using shRNAs would affect Pax2 expression in wild type MEF cells. The results showed that substantial knockdown of EZH2, SUZ12, and WT1 obviously increased Pax2 expression in wild type MEF cells, respectively (Fig. 4*E*). Control vector and constructs expressing each of the two distinct shRNAs that specifically target WT1 were generated and stably transfected into MEF cells. Both WT1 shRNA1 and 2 substantially increased *Pax2* mRNA level as shown by qRT-PCR (Fig. 4*F*), and it is correlated with the protein level (Fig. 4*E*). We further determined whether WT1 regulates *Pax2* transcription through H3K27 trimethylation. The ChIP results showed that both WT1 shRNA 1 and 2 notably decreased the H3K27me3 level at *Pax2* promoter loci (Fig. 4*G, lanes 6* and *9*). These results support that WT1 and PcG proteins are essential for H3K27me3 histone modification and suppression of Pax2 expression by menin.

WT1 Interacts with PcG and DNMT1—PRC2 is thought to repress gene transcription by catalyzing di- and trimethylation of H3K27 (11). To determine better whether WT1 has a role in regulating target genes in concert with PcG, we determined the potential protein-protein interaction between WT1 and PcG using co-IP. Cell extracts from MEFs were immunoprecipitated with a control antibody (IgG) or anti-WT1 antibody, and the result showed that endogenous WT1 co-precipitated with SUZ12 and EZH2 (Fig. 5*A*). As a control, IgG failed to pull down WT1 (Fig. 5*A*). Interaction between PRC2 and WT1 was further confirmed by reverse co-IP of endogenous WT1 with anti-EZH2 and SUZ12 antibodies (Fig. 5, *B* and *C*). We next sought to determine whether menin associates with the WT1-PcG complex. However, we failed to detect menin binding to both EZH2 and PcG with co-IP and reverse co-IP (Fig. 5*D*). EZH2 specifically methylates H3K27; knockdown of EZH2 resulted in down-regulated global H3K27me3 (19). To determine whether WT1 affects H3K27me3, the WT1 and EZH2 cDNA were
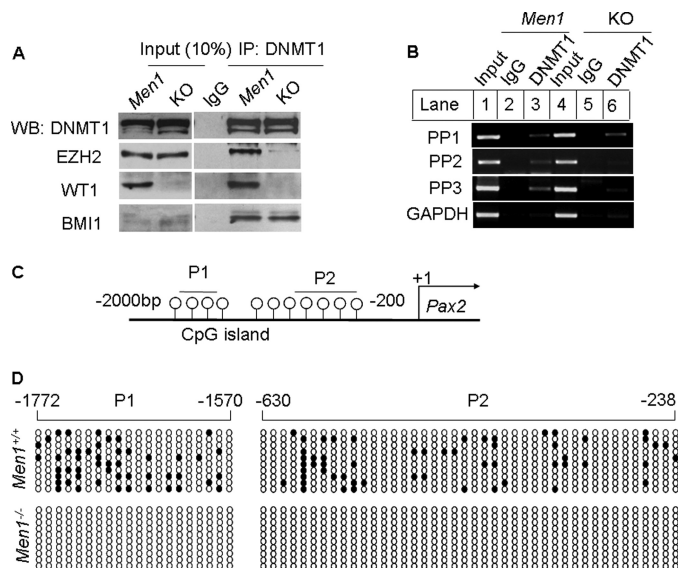
transfected into 293T cells, respectively. The Western blot results show that ectopic expression of either WT1 or EZH2 slightly increased global H3K27me3 ([supplemental Fig. S5](#)).

PcG-mediated gene silencing is usually associated with DNA hypermethylation (35). Our results showed that the PcG complex proteins specifically interacted with DNMT1 (Fig. 5, *A–C*). Notably, we found that WT1 also interacted with DNMT1 (Fig. 5, *A–C*). Consistent with these observations, the reverse co-IP also showed that endogenous EZH2, BMI1, and WT1 were co-immunoprecipitated with endogenous DNMT1 (Fig. 6*A*). We further performed ChIP assays to detect whether DNMT1 is associated with the *Pax2* locus. The results showed that DNMT1 bound to the *Pax2* promoter (PP2 and PP3) in the *Men1*-reexpressing MEFs, but the *Men1* excision abrogated DNMT1 binding to the *Pax2* locus (Fig. 6*B, lanes 3* and *6*). Notably, down-regulated WT1 by *Men1* excision reduced interaction between DNMT1 and EZH2 (Fig. 6*A*) but did not affect the interaction between PRC2 proteins, such as EZH2 and SUZ12 (Fig. 5, *B* and *C*). These results suggest that WT1 mediates interaction between DNMT1 and PRC2. We also carried out bisulfate genomic sequencing of *Pax2* promoter in *Men1*-null and *Men1*-reexpressing MEFs. We found that many CpG of the promoter of *Pax2* were methylated in *Men1*-reexpressing MEFs, whereas there was no methylated CpG in *Men1*-null MEFs (Fig. 6*C*), suggesting that function of menin/WT1 is also involved in DNA methylation of the promoter. Collectively, these results indicate that WT1 is a specific cofactor for menin-dependent and PcG-mediated H3K27 trimethylation and DNA hypermethylation for gene silencing.

DISCUSSION

Epigenetic gene regulation plays essential roles for development of organisms and cell differentiation. Several studies have demonstrated a crucial role for menin in MLL-mediated H3K4 histone modifications (9, 16, 18, 36). However, the potential role of menin in PcG-mediated gene regulation has not been addressed. In a previous report, we found that menin represses transcription of target genes via epigenetic H3K27me3 modification (19), but the precise biochemical mechanism is poorly understood. PcG proteins regulate expression of a large number of factors that determine cell fate decisions during development. Among them, *Pax* family genes play a key role in formation of tissues and organs during embryonic development (24). Pax2 is the third paired domain protein present in the endocrine pancreas and may be implicated in the determination of the relative proportion of endocrine and exocrine tissues during pancreas development (37, 38).

In the present study, menin specifically represses *Pax2* transcription and expression, which is associated with H3K27me3 tail modification at the *Pax2* promoter. This is consistent with the previous ChIP-sequencing results that SUZ12 protein binds to the promoter of the *Pax2* gene (25). In addition, *Men1* excision abrogated binding of PcG proteins and DNMT1, but increased RNA polymerase II to *Pax2* promoter in MEFs. This observation is consistent with our previous findings that menin represses pleiotrophin gene transcription via PcG-dependent H3K27me3 at its promoter (19). These findings suggest that menin participates in PcG complex-mediated gene silencing and histone tail modification. Further investigation failed to detect that menin directly bound to PcG complex. It is likely that menin regulates H3K27 modification indirectly, depending on other factors that affect recruitment or functions of PRC2.

The Wilms tumor suppressor gene, *WT1*, encodes a zinc finger protein capable of high affinity binding to DNA/RNA (39). Many studies have demonstrated that WT1 plays a key role in regulating cell growth and differentiation in the urogenital system (20, 40, 41) and endocrine organs (33). Several genes have been identified as early markers of kidney progenitor cells, including *WT1* (41), *Pax2* (42), and *Hox11* paralog group (43), and these genes are required for proper differentiation of the metanephric kidney. The *Pax2* gene contains WT1 binding sites, and WT1 could directly repress *Pax2* transcription during normal kidney development (32). Based on these observations, we hypothesize that menin represses *Pax2* transcription by regulating WT1. We found that menin interacted with WT1 in the nucleus and significantly up-regulated protein levels of WT1, but did not affect the WT1 mRNA level. It raised a possibility that menin regulates WT1 expression via posttranscriptional modification. Our results exclude that this regulation was dependent on HtrA2, a protease that specifically that cleaves WT1 at multiple sites (34). Instead, menin may bind to WT1 and protects its degradation from other hitherto unidentified specific cleavage enzyme. Alternatively, it is also possible that menin interferes with translation of WT1 mRNA or WT1 degradation involving proteasomes or lysosomes. These various mechanisms remain to be investigated. Precise expression of WT1 contributed to normal kidney development (44). Importantly, we found that *Men1* excision reduced expression of WT1 in kidney of the $MEN1^{+/-}$ mice, suggesting a potential role of menin in regulating renal development and kidney disease.

Recently, some novel proteins have been identified as associating with PRC2 and stimulating its H3K27-methylating enzymatic activity. For example, Jumonji and ARID domain-containing protein JARID2 is a newly discovered key regulator for methylation of H3K27, and it interacts with PcG proteins and binds to DNA in ES cells (25, 45). Currently, little is known as to whether WT1 participates in histone modifications. WT1 binds to DNA through its C terminus that contains four Cys-His zinc finger domains. Metsuyanim *et al.* have reported that sequential epigenetic alterations involving PcG activation and epigenetic modifications of the nephric progenitor genes, including *WT1*, *Pax2*, and *six2*, led to tumor initiation and progress. These observations suggest a potential role for WT1 in DNA binding, chromatin modifications, and repression of gene transcription.

Our ChIP results indicate that WT1 directly bound to the *Pax2* promoter and increased H3K27 trimethylation. Co-IP and reverse co-IP also demonstrate that WT1 interacts with PcG complex, including EZH2 and SUZ12. Ectopic expression of WT1 repressed Pax2 expression and increased H3K27me3 level at the *Pax2* promoter in *Men1*-null MEFs. In addition, *Men1* ablation and WT1 down-regulation lead to increased RNA polymerase II but decreased DNMT1 recruitment to the *Pax2* promoter, unraveling an important mechanism for menin and PcG-mediated gene silencing (35). These observations, for the first time, demonstrate that menin-WT1 mediated silencing of gene expression in part depends on PcG-mediated H3K27me3. How WT1 might interact with PRC protein members remains to be determined.

Collectively, these studies have unraveled crucial mechanisms whereby menin represses *Pax2* transcription, uncovering a novel epigenetic mechanism involving H3K27me3 and WT1 by menin. These findings suggest that WT1 protein exerts an important, yet previously unappreciated function in regulating H3K27 histone modifications and repressing gene expression. These results also suggest a biological function of menin-WT1 regulated *Pax2* signaling in endocrine neoplasia and renal disease.

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